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Catabolism of L-Tyrosine by the Homoprotocatechuate Pathway in Gram-Positive Bacteria

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A metabolic pathway for L-tyrosine catabolism involves 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid) as substrate for fission of the benzene nucleus. Cell extracts of an organism tentatively identified as a *Micrococcus* possessed the enzymes required for degrading homoprotocatechuate to succinate and pyruvate, and stoichiometry was established for several of these reactions. When the required coenzymes were added, cell extracts degraded L-tyrosine to the ring-fission product of homoprotocatechuate 2,3-dioxygenase and also converted 4-hydroxyphenylpyruvic acid into 4-hydroxyphenylacetic acid. This compound, in turn, gave stoichiometric amounts of the ring-fission product of homoprotocatechuate by the action of a nicotinamide adenine dinucleotide phosphate-dependent 3-hydroxylase coupled with homoprotocatechuate 2,3-dioxygenase. Evidence is presented that this route for L-tyrosine catabolism is taken by five other gram-positive strains, including *Micrococcus lysodeikticus* and a species of *Bacillus*. Five other gram-positive bacteria from other genera employed the alternative homogentisate pathway.

Studies of utilization of tyrosine as carbon source for bacterial growth have hitherto been restricted to a few strains that use the homogentisate pathway (3, 7); thus, homogentisate 1,2-dioxygenase (EC 1.13.11.5) was purified and crystallized from *Pseudomonas fluorescens* adapted to tyrosine (1). The pathway is modified in a *Bacillus* which lacks the isomerase for the ring-fission product of homogentisate (R. L. Crawford, personal communication). A similar modification in the gentisate pathway of catabolism has also been observed (10) and entails the elaboration of an enzyme system for metabolizing D-malate (8, 9). However, the existence of a pathway for degrading tyrosine, completely separate from that involving homogentisate, was suggested by the observation (11) that a species of *Pseudomonas* when grown with tyrosine oxidized 3,4-dihydroxyphenylacetic acid (homoprotocatechuate). We have made similar observations for several gram-positive species, and we now report detailed studies to show that one of these strains uses the enzymes that catalyze the homoprotocatechuate sequence of reactions (16) for degrading tyrosine. A complete scheme for this new route of catabolism is shown in Fig. 1.

MATERIALS AND METHODS

Organisms and cell extracts. Strains able to utilize L-tyrosine for growth were selected from our laboratory collection of gram-positive organisms. The following such strains were originally isolated from soil in St. Paul, Minn., by virtue of their ability to grow with 4-chlorobenzoic acid as carbon source (P. J. Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, O2, p. 192) and were tentatively assigned to the genus *Micrococcus*: strains 1/2, 3/2, 6/2, and S9. We have used strain S9 in most of this work and are indebted to Sheue L. Keenan for providing us with a mutant of this organism that was obtained during the course of isolating mutants defective in chlorobenzoate metabolism. Mutant strain S9/17, when grown on nutrient agar slants, formed an intense yellow color which was shown to arise from the partial degradation of the tyrosine in the growth medium. Furthermore, this material also accumulated on adding tyrosine to cultures of the mutant growing at the expense of succinate and, when examined, was shown to possess the spectroscopic and biochemical properties of the ring-fission product of homoprotocatechuate (16).

Other organisms used were: *Micrococcus lysodeikticus*, British NCIB 9278; *Mycobacterium rhodochrous*, NCIB 9784, an organism that grows with camphor and was originally designated strain T1 (4); *Nocardia globerula* CL-1, isolated from soil by enrichment with cyclohexanol (13); *Nocardia rhodnii* from the collection of Muriel Rhodes, University College of Wales, Aberystwyth, U.K.; *Bacillus* 8, isolated from pasteurized soil in St. Paul, Minn., by enrichment with 4-hydroxyphenylpropionic acid (R. L. Crawford and P. J. Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, O3, p. 192); a species of *Corynebacterium* isolated from soil by Rosenberg and Holmes (14) by enrichment with 3-tetrahydrofuran l; and an unidentified gram-positive orga-

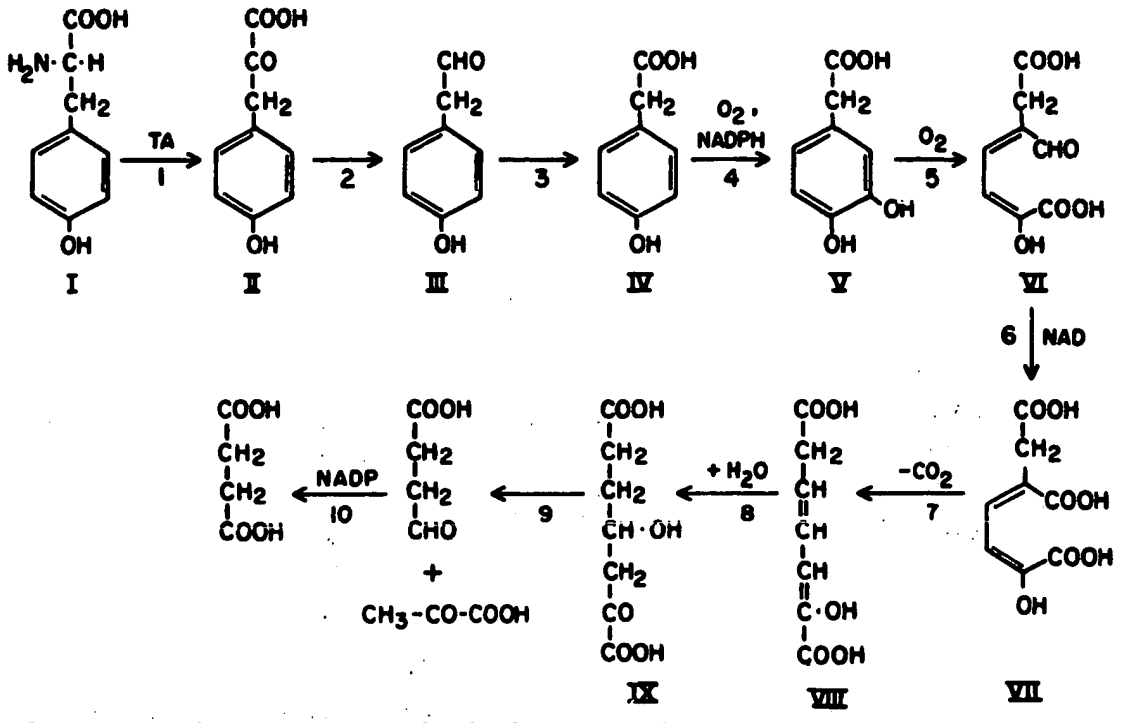

FIG. 1. *Proposed reaction sequence for the degradation of* L-*tyrosine to pyruvic and succinic acids. Reactions 4 through 10 are those previously described for strains of Acinetobacter* and *Pseudomonas putida (16).*

nism, Resorcinol 5/2, which was isolated from soil by enrichment with resorcinol and closely resembles the above mentioned *Corynebacterium* in color and morphology. Cultures of all these organisms were grown with aeration at 30 C in the basal medium of Sparnins et al. (16) modified to contain (per liter) 2 ml, instead of 4 ml, of the stock solution of salts specified by Rosenberger and Elsden (15). The carbon source was either L-tyrosine (0.04%) or L-tyrosine (0.04%) plus succinic acid (0.1%), and in addition all media contained yeast extract (0.005%) and Casamino Acids (0.005%). Cell extracts were prepared in 0.1 M K^+-Na^+ phosphate buffer, pH 7.0, from frozen cell pastes crushed in a Hughes bacterial press (5, 17). For use in certain experiments, 1 volume of cell extract was dialyzed for three successive periods of 30 min against 100 volumes of 0.1 M phosphate buffer, pH 7.0.

Measurement of gas exchanges and enzyme activities. Warburg respirometry was used as previously described (16) to measure rates of oxidation of substrates by intact cells and also to measure the simultaneous evolution of carbon dioxide and consumption of oxygen observed when a cell extract degraded 4-hydroxyphenylpyruvate. In this experiment, flasks contained 4 μmol of substrate, 1 μmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 6 mg of dialyzed cell extract protein in a total volume of 3 ml of 0.1 M K^+-Na^+ phosphate buffer, pH 7.0. On all other occasions, rates of oxygen consumption catalyzed by cell xtracts were measured by means of an xygen electrode operated as described previ usly (17). The reaction sequence proposed (Fig. 1) gives rise to succinic semialdehyde and pyruvate. Extracts contained a specific nicotinamide adenine dinucleotide phosphate (NADP)-dependent dehydrogenase for succinic semialdehyde, which permitted the determination of this metabolite irom the increase of absorbancy measured at 340 nm when NADP was added. Pyruvate was also determined from the decrease in absorbancy at this wavelength when reduced nicotinamide adenine dinucleotide (NADH) was oxidized on adding lactate dehydrogenase (16). Methods used for chromatography and identification of the 2,4-dinitrophenylhydrazones of metabolites and also for examination of trimethylsilyl derivatives by mass spectrometry were described by Sparnins et al. (16).

Materials. Samples of 5-carboxymethyl-2-hydroxymuconic semialdehyde (compound VI, Fig. 1) and 5-carboxymethyl-2-hydroxymuconic acid (compound VII) were prepared from homoprotocatechuate (compound V) by the action of suitably treated cell extracts of *Pseudomonas* U or *Pseudomonas* T (16). L-β-3,4-Dihydroxyphenylalanine, homogentisic, homoprotocatechuic, 4-hydroxyphenylacetic, and 4-hydroxyphenylpyruvic acids were from Sigma Chemical Co., St. Louis, Mo. L-Tyrosine was from Mann Research Labs., Inc., New York, N.Y. 4-Hydroxy-2-ketopimelic acid (compound IX) was synthesized by P-T. Leung (12).

RESULTS AND DISCUSSION

Oxidation of metabolites by intact cells f strain S9. Washed-cell suspensions of strain S9

grown with L-tyrosine readily oxidized compounds I, II, IV, and V of Fig. 1, namely L-tyrosine, 4-hydroxyphenylpyruvate, 4-hydroxyphenylacetate, and homoprotocatechuate, respectively (Table 1). L-β-3,4-Dihydroxyphenylalanine was oxidized at a slower rate that was probably due to nonspecific oxidation. Homogentisate was oxidized at about one-sixteenth of the rate for homoprotocatechuate. Cells grown with succinate were not induced to metabolize the compounds of Fig. 1.

Conversion of tyrosine into compound VI. When incubated with an extract of L-tyrosine-grown cells of strain S9, L-tyrosine was converted into a compound having spectral characteristics the same as those of 5-carboxymethyl-2-hydroxymuconic semialdehyde (compound VI). For this comparison, a sample of the compound was obtained from homoprotocatechuate by the action of a cell extract of 4-hydroxyphenylacetate-grown *Pseudomonas* T (16). The conversion of tyrosine into compound VI was entirely dependent upon the addition of 2-ketoglutarate and NADPH to the incubation mixture and was monitored by the increase in absorbancy at 380 nm (16). When nicotinamide adenine dinucleotide (NAD) was added (Fig. 2) the absorbancy decreased immediately, and a slow accumulation of pyruvate could be demonstrated spectrophotometrically upon additions of NADH and lactate dehydrogenase. These observations are consistent with the requirement of 2-ketoglutarate for transamination of tyrosine (reaction 1, Fig. 1), of NADPH for reaction 4, and of NAD for oxidation of compound VI by reaction 6.

Metabolism of compound VI. In the presence of NAD, cell extracts of strain S9 readily attacked a sample of this compound prepared from homoprotocatechuate using an extract of *Pseudomonas* T. Furthermore, cell extracts of the mutant of strain S9 that formed compound VI from tyrosine were shown to accumulate compound VI from homoprotocatechuate (Fig. 3). No further reaction occurred when NAD was added, confirming that the mutant lacked enzyme 6 of Fig. 1; however, when extract of 4-hydroxyphenylacetate-grown *Acinetobacter* (16) was added to the reaction mixture containing NAD, compound VI was metabolized immediately (Fig. 3). The same result was obtained by adding an extract of tyrosine-grown wild-type S9. When this extract was dialyzed, compound VIII was accumulated from hom protocatechuate, as shown by the ultraviolet absorption spectra of the solution in acid and base (16). In this experiment, a cuvette contained 1

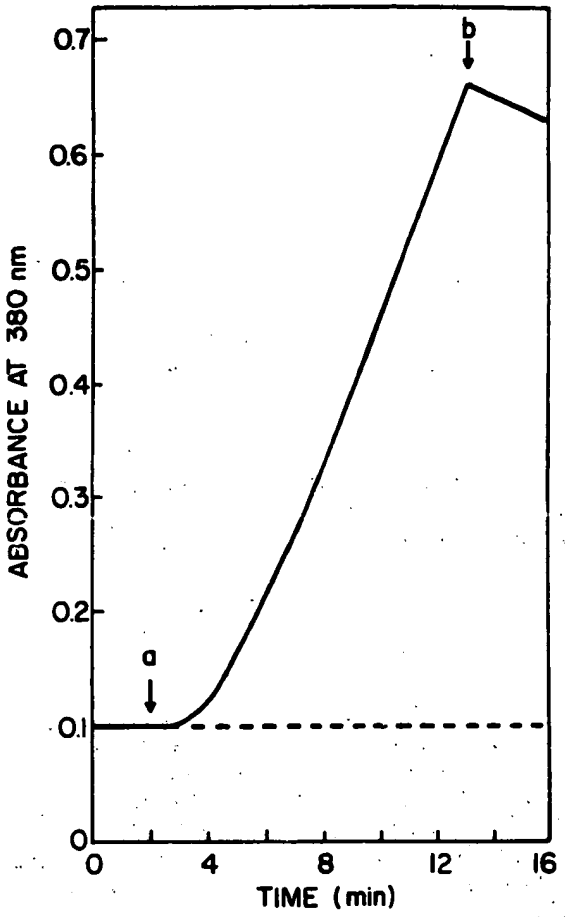

FIG. 2. *Conversion of L-tyrosine into compound VI (λ_{max} at 380 nm). The cuvette contained (in 1 ml) 100 μmol of phosphate buffer, pH 7.0, 1 μmol of L-tyrosine, 0.1 μmol of NADPH, and 0.5 mg of crude cell extract protein from wild-type strain S9 grown with tyrosine. At (a) 1 μmol of 2-ketoglutarate was added, and at (b) 0.05 μmol of NAD was added. The dashed line shows omission of either 2-ketoglutarate or NADPH.*

TABLE 1. *Rates of oxidation by washed cells of strain S9*

Compound oxidized	Oxygen uptake[a]	
	Tyrosine[b]	Succinic acid[b]
L-Tyrosine	6.6	0.1
Homoprotocatechuic acid	8.1	0.1
4-Hydroxyphenylpyruvic acid	3.7	0.1
4-Hydroxyphenylacetic acid	5.4	0.2
L-β-3,4-Dihydroxyphenylalanine	1.5	<0.1
Homogentisic acid	0.5	<0.1
Succinic acid	0.5	6.5

[a] Expressed as microliters of O_2 per minute and measured after subtraction of endogenous respiration (0.40 μl of O_2 per min). Warburg cups contained, in 3 ml of 0.1 m phosphate buffer (pH 7.0), 4 mg (dry weight) of cells and 4 μmol of substrate.

[b] Compound on which cells were grown.

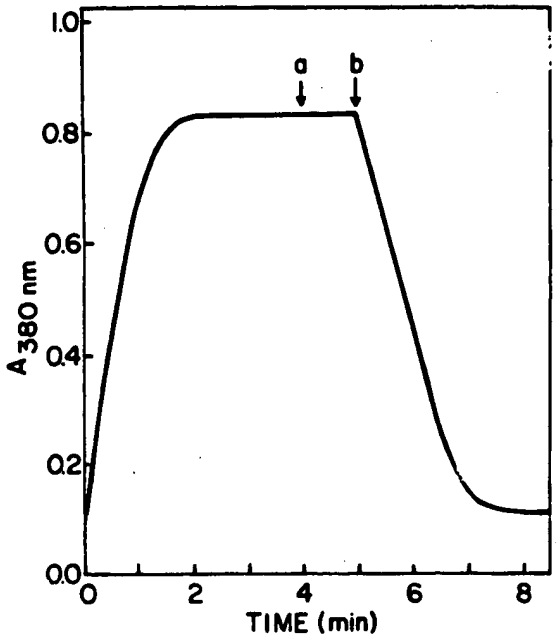

FIG. 3. *Formation of compound VI from homoprotocatechuate and subsequent degradation. The cuvette contained 1 ml of 0.1 M phosphate buffer, pH 7.5, and 20 nmol of homoprotocatechuate. The reaction was started by addition of 0.2 mg of cell extract protein from a mutant of strain S9. At (a) 20 nmol of NAD was added, and at (b) 0.15 mg of cell extract protein of Acinetobacter grown with 4-hydroxyphenylacetic acid was added. The mutant strain was grown in a succinate medium containing L-tyrosine.*

ml of 0.1 M phosphate buffer, pH 7.0, and 0.03 μmol of homoprotocatechuate. When dialyzed extract of wild-type strain S9 (0.1 mg of protein) was added, compound VI was formed and disappeared on addition of 0.03 μmol of NAD with the transient formation of VII (λ_{max} at 300 nm) and the accumulation of compound VIII, having λ_{max} at 274 nm, pH 7.0 (16). When the cell extract was applied to a column of Ultrogel AcA 34 as previously described (17), a fraction was obtained that accumulated only compound VII, presumably because this treatment removed Mg^{2+} ions required in the catalysis of reaction 7 (Fig. 1) (16).

The further metabolism of compound VII was studied with samples of this compound previously prepared from homoprotocatechuate by the action of a heat-treated extract of *Pseudomonas* U (16). A crude cell extract of strain S9 grown with tyrosine converted 1 mol of compound VII into approximately 1 mol each of pyruvate and succinic semialdehyde, determined as described in Materials and Methods (Table 2). The identity of the products was confirmed by chromatography of their 2,4-dinitrophenylhydrazones. Chemically synthesized (12) 4-hydroxy-2-ketopimelic acid (compound IX) was likewise converted by this cell extract into equimolar amounts of pyruvate and succinic semialdehyde.

Metabolism of 4-hydroxyphenylpyruvate and 4-hydroxyphenylacetate. 4-Hydroxyphenylpyruvic acid (compound II) is shown as undergoing decarboxylation to 4-hydroxyphenylacetaldehyde (compound III) which is oxidized to 4-hydroxyphenylacetic acid (compound IV) (Fig. 1). Although we have not shown the formation of compound III, Warburg respirometry was used as described in Materials and Methods to demonstrate the conversion of 4.0 μmol of 4-hydroxyphenylpyruvate into compound VI with the concomitant uptake of 7.5 μmol of oxygen and evolution of 4.4 μmol of carbon dioxide. These values for carbon dioxide yield are in approximate agreement with the scheme of Fig. 1, as was the requirement of this reaction for added NADPH. When the latter was omitted, compound II gave rise to 4-hydroxyphenylacetic acid which was identified by mass spectrometry of its trimethylsilyl derivative. The NADPH-dependent oxidation of 4-hydroxyphenylacetic acid was examined by means of an oxygen electrode. The reaction vessel contained, in 1.5 ml of 0.1 M phosphate buffer, pH 6.5, dialyzed extract of strain S9 (0.25 mg of protein) and different amounts of 4-hydroxyphenylacetic acid (Table 3). Reactions were started by adding 200 nmol of NADPH, and when uptake of oxygen ceased the amounts of compound VI formed were determined spectrophotometrically at 380 nm after adjusting the pH to 7.0 (and using a value of ϵ = 30,000). The observed stoichiometry is approximately that required by the scheme of Fig. 1.

TABLE 2. *Stoichiometry of conversion of compound VII to pyruvate and succinic semialdehyde by an extract of strain S9*

Compound VII (μmol)	Pyruvate (μmol)	Succinic semialdehyde (μmol)
18	14	16
27	28	26
36	35	31
45	41	46

TABLE 3. *Stoichiometry of oxidation of 4-hydroxyphenylacetic acid (4-HPA) by a dialyzed extract of strain S9*

4-HPA (nmol)	Uptake of O_2 (nmol)	Compound VI formed (nmol)	Uptake of O_2/4-HPA used
25	52	26	2.05
30	58	28	1.95
40	81	36	2.02
50	100	43	2.00

TABLE 4. *Dioxygenase activities of cell extracts of vari us organisms grown with tyrosine*

Organism	Sp act (nmol/min per mg of protein)	
	Homoprotocatechuate 2,3-dioxygenase	Homogentisate 1,2-dioxygenase
Strain S9	150	<5
Strain 1/2	48	7
Strain 3/2	97	18
Strain 6/2	52	5
Bacillus 8	130	<5
Micrococcus lysodeikticus	143	7
Corynebacterium	8	426
Nocardia globerula CL-1	8	318
Nocardia rhodnii	9	160
Mycobacterium rhodochrous	1	480
Resorcinol 5/2	2	335

Dioxygenase activities of various organisms. The oxygen electrode was used as described to determine the specific activities of cell extracts of organisms after growth with tyrosine. *Micrococcus lysodeikticus*, *Bacillus* 8 and strains S9, 1/2, 3/2, and 6/2 gave high specific activities for homoprotocatechuate 2,3-dioxygenase and low activities for homogentisate 1,2-dioxygenase, whereas the remainder of the strains examined showed the reverse pattern (Table 4). It may, therefore, be assumed that the former group employed the homoprotocatechuate pathway for tyrosine catabolism and the latter, the homogentisate pathway. In the case of *Bacillus* 8 this conclusion was confirmed by showing that intact cells readily oxidized L-tyrosine, 4-hydroxyphenylacetic acid, and homoprotocatechuic acid but not homogentisic acid. Furthermore, cell extracts of this organism converted homoprotocatechuate into pyruvate, on addition of NAD, with the expected stoichiometry, and they also degraded compounds VI and VII.

Previous work demonstrated that the enzymes for homoprotocatechuate degradation can be induced in *Pseudomonas acidovorans*, an organism that employs the homogentisate pathway for degrading 4-hydroxyphenylacetic acid and tyrosine. In the present investigation we have shown that several gram-positive organisms employ these enzymes in tyrosine catabolism. It may be observed that homoprotocatechuate has been shown to be an intermediate in the degradation of *dl*-synephrine by *Arthrobacter* (6) and of tyramine by *Aerobacter aerogenes* (2).

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